

Development of Peptide Antagonists That Target Estrogen Receptor β -Coactivator Interactions

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The biological actions of estrogen are manifest through two genetically distinct estrogen receptors (ER α and ER β) that display nonidentical expression patterns in target tissues. The phenotypic alterations in response to estrogens in mice disrupted for either or both of these receptors are not identical, suggesting that each subtype plays a unique role in ER-action. However, the lack of subtype-specific agonists and antagonists has made it difficult to define the processes that are regulated by ER α and/or ER β . Previously, we have reported the identification and characterization of a series of LXXLL-containing peptide antagonists that block estrogen signaling by preventing the association of ER α with required coactivators. As expected, given the similarity of the coactivator binding pockets among nuclear receptors, most of the peptide antagonists identified inhibited the activity of multiple receptors. However, by altering sequences flanking the core LXXLL motif, some receptor selectivity was afforded. Building on this observation, we have screened combinatorial phage libraries, expressing peptides in the format X₇LXXLLX₇, for peptides that interact in a specific manner with ER β . Using this approach, a series of highly specific, potent peptide antagonists have been identified that efficiently inhibit ER β -mediated estrogen signaling when introduced into target cells. Interestingly, in cells where both ER subtypes were expressed, these ER β antagonists were capable of attenuating ER action, suggesting that ER α and ER β do indeed form functional heterodimeric complexes. We believe that suitably formulated versions of these peptides can be used to study ER β action *in vitro* and *in vivo*. In addition, the unanticipated specificity of the peptides identified should serve as an impetus to investigate the use of this approach to develop peptide antagonists of other nuclear receptors and unrelated

transcription factors. (Molecular Endocrinology 14: 2010–2023, 2000)

INTRODUCTION

The human estrogen receptor (ER) belongs to the nuclear receptor superfamily of ligand-inducible transcription factors (1), whose members include the receptors for steroids, thyroid hormone, retinoic acid, vitamin D, and orphan receptors for which no ligands have yet been identified. The mechanism of action of ER is similar to that of other nuclear receptors (2). In the absence of hormone, the receptor is sequestered within the nuclei of target cells in an inactive state. The binding of ligand induces an activating conformational change within ER, an event that permits the receptor to interact with transcriptional coactivators such as steroid receptor coactivator 1 (SRC-1) and glucocorticoid receptor interacting protein 1 (GRIP1) (3, 4), and which facilitates the association of the resulting complex with specific DNA response elements (EREs) located within the regulatory regions of target genes. Depending on the promoter context, the DNA-bound receptor can then exert either a positive or negative effect on target gene transcription (2, 5).

Until recently it was thought that all of the biological actions of estrogens and antiestrogens were manifest through a single receptor located within target cell nuclei. However, the identification of a second estrogen receptor, ER β (6, 7), has indicated that estrogen signaling is more complex. The two ER subtypes, ER α and ER β , share extensive amino acid similarity in their ligand- and DNA-binding domains, but minimal homology within their amino-terminal regions. Not surprisingly therefore, these receptors exhibit similar, but not identical, ligand binding characteristics (8) and interact with the same DNA response elements. The most obvious difference between the two receptors is that ER α is a more efficient activator of ERE-containing genes than ER β under most circumstances (7, 9–11). In addition, it has been noted that ER β can interact in a constitutive manner with target promoters

and can attenuate the ligand-activated transcriptional activity of ER α (11). Thus, in cells where both receptors are expressed, overall estrogen responsiveness is reduced.

In parallel with studies performed *in vitro*, the creation and characterization of mice in which either ER α and/or ER β have been disrupted (α ERKO, β ERKO and $\alpha\beta$ ERKO, respectively) have demonstrated that the two receptors are not functionally equivalent and that each subtype plays a unique role in ER action *in vivo* (12, 13). However, it is clear that in addition to these mouse models, there is a need for subtype-selective agonists and antagonists that will permit the transient manipulation of receptor function in intact animals. Consequently, to complement the efforts of others who are engaged in screening for small molecules that interact with the ligand-binding pockets of ER α and ER β (15), we have undertaken a novel approach to develop subtype-specific antagonists that inhibit ER β action in a manner distinct from known antiestrogens.

All of the currently available ER antagonists function by 1) binding to the receptor ligand-binding domain, thereby blocking agonist access, and 2) inducing a conformational change within the receptor that prevents it from interacting efficiently with transcriptional coactivators such as SRC-1, GRIP1, and amplified in breast cancer 1 (AIB-1). Specifically, it has been shown that agonist binding to the receptor induces a conformational change that permits the formation of a hydrophobic pocket (16, 17), enabling the receptor to interact with the LXXLL motif contained within the receptor interaction domains of most of the validated coactivators (18, 19). The conformational changes induced in ER upon antagonist binding do not permit coactivator recruitment (19). Clearly, the most direct method of inhibiting ER function would be to develop drugs that bind directly to the coactivator binding pockets within ER α or ER β and block coactivator recruitment. Given that the coactivator binding pockets in ER α , ER β , and other nuclear receptors are structurally similar and that most of the known coactivators do not appear to demonstrate receptor selectivity, it was not obvious that the receptor-cofactor binding pocket was a bona fide drug target. However, we have recently identified a series of LXXLL-containing peptides that interact very well with the coactivator binding pocket of ER β , but which demonstrate distinct preferences in their ability to interact with other receptors (20). Thus, all LXXLL motifs are not functionally equivalent. Building on this observation, in the current study we have identified LXXLL-containing peptides that interact specifically with ER β and inhibit its transcriptional activity. We believe that these novel peptide antagonists will serve as useful tools to evaluate the role of ER β in estrogen signaling. In addition, we anticipate that the general approach used to obtain these ER β antagonists can be applied to the development of peptide antagonists of other nuclear receptors and unrelated transcription factors.

RESULTS

Affinity Selection of ER β -Binding LXXLL-Containing Peptides Using Phage Display

A critical step in ER action is the ligand-dependent recruitment of transcriptional coactivators to target gene promoters. Antiestrogens manifest their inhibitory activities by altering ER structure and independently blocking cofactor binding (19, 22). In this study, the feasibility of targeting ER-coactivator interactions directly as a mechanism of developing human ER β -specific antagonists was evaluated. Using combinatorial phage display technology (21), we created and screened a phage library that expressed 19-mer peptides containing a central fixed Leu-X-X-Leu-Leu motif flanked by seven random amino acids on each side. Since we used an NNK nucleic acid format in the construction of this library it has a theoretical complexity of 1.2×10^{24} . However, since our library contained only 10^8 independent clones, we are only surveying a fraction of the potential LXXLL motifs that can interact with ER β . This random LXXLL library was screened for phage that bound to ER β either in the absence or presence of estradiol, and 70 of the most avid interactors identified were brought forward for further analysis. A secondary enzyme-linked immunosorbent assay (ELISA) was used to confirm the ER β -binding characteristics of the plaque-purified phage. Cross-screening, using a similar approach, revealed that 37 of the phage identified bound to both ER subtypes whereas 33 interacted selectively with ER β . The latter subset of phage expressing LXXLL-containing peptides were brought forward for further analysis.

Characterization of the ER β -Selective LXXLL-Containing Peptides in Intact Cells

We performed a mammalian two-hybrid assay to assess the ability of the peptide sequences identified by phage display to interact selectively with ER β in intact cells. Each of the 33 ER β -selective peptides identified *in vitro* were expressed as a yeast Gal 4 DNA-binding domain (Gal4DBD) fusion protein and tested for their ability to interact with ER α and/or ER β expressed as fusions to the VP16 activation domain. Expression of the peptide-fusions was confirmed by Western immunoblotting (data not shown). A Gal4DBD-fusion of the NR-box of the ER coactivator SRC-1 (containing three LXXLL motifs), shown previously to interact with both ER α and ER β , was used in this assay as a positive control. When analyzed in the two-hybrid assay, it was found that 15 of the 33 peptides studied interacted with ER β , but not ER α . Representative examples are shown in Fig. 1 of those peptides that were isolated in the screen with the apo-ER β (Fig. 1A), and those identified with the agonist-liganded receptor (Fig. 1B). Interestingly, several of the peptides were able to bind ER β in the absence of ligand, suggesting either that a portion

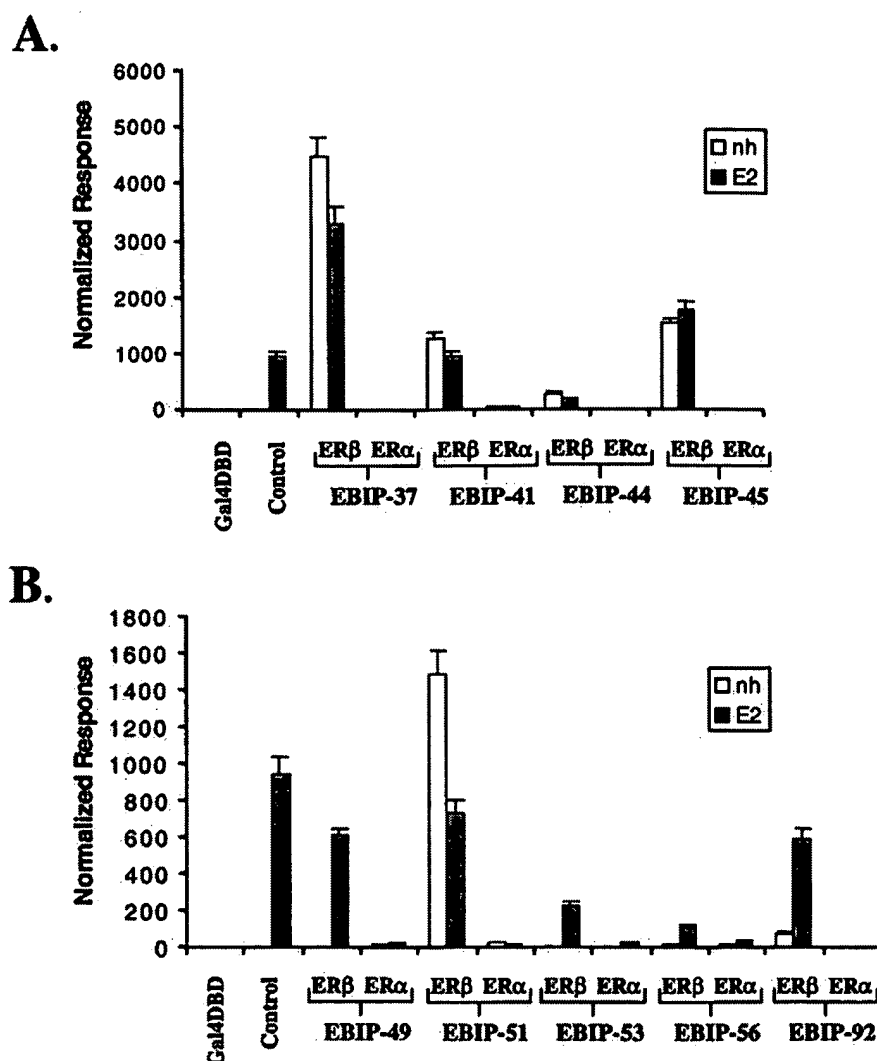


Fig. 1. Evaluation of ERβ-Selective LXXLL-Containing Peptides in Intact Cells

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERα or ERβ in mammalian cells. Each ER expression construct includes the VP16 activation domain sequence fused 5' to the complete coding sequence for the human ERα or ERβ. A, Analysis of the interaction of peptides that were isolated in the screen with apo-ERβ (four representative peptides are shown). B, Analysis of the interaction of peptides that were isolated in the screen with agonist-liganded ERβ (five representative peptides are shown). HepG2 cells were transiently transfected with the pVP16-ERα expression vector or the pVP16-ERβ expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. The SRC-1 (NR-box)-Gal4DBD fusion was used as a control. Cells were induced with vehicle (nh) or 10^{-7} M 17β-estradiol (E₂) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

of the ERβ aporeceptor resides in an active conformation, or that the binding of LXXLL-containing motifs to the receptor may facilitate activation. Using the mammalian-two hybrid assay, it was also shown that the introduction of inactivating point mutations into the AF-2 region of ERβ (11) completely abolished the interaction of each peptide with the receptor (see Fig. 7A, below). These results suggested that the peptides were targeting the coactivator binding pocket, and thus would be able to antagonize ERβ transcriptional activity.

ERβ-Selective LXXLL Peptides Display Hormone Specificity

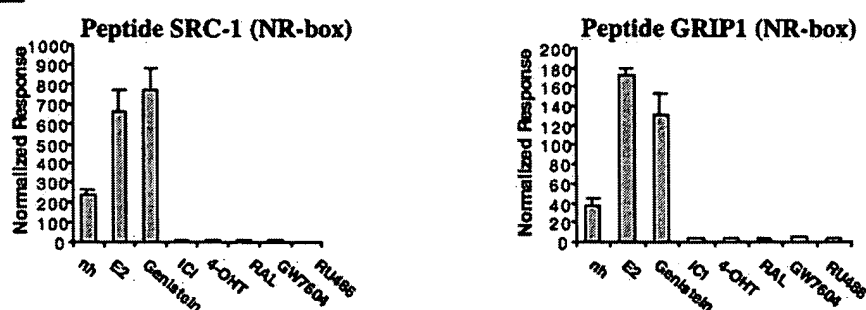
The interaction of LXXLL-containing coactivators such as SRC-1 or GRIP1 with ERα and ERβ has been shown to require agonist activation of the receptor (3–5). The surprising finding that some LXXLL-containing peptides can interact with apo-ER begged a re-evaluation of the role of ligand in regulating ERβ-LXXLL interactions. This was accomplished using the mammalian two-hybrid assay to examine the effect of

different ER ligands on peptide binding. As expected, the interactions between the Gal4DBD-SRC-1, and Gal4DBD-GRIP1 NR-box fusions and ER β were enhanced by the addition of the agonists 17 β -estradiol and genistein (Fig. 2A). However, administration of antiestrogens alone antagonized the basal receptor-peptide interactions. Interestingly, both of the control peptide fusions showed significant levels of hormone-independent interaction with ER β . A similar observation was made *in vitro*, where glutathione-S-transferase (GST)-pull down assays revealed that both

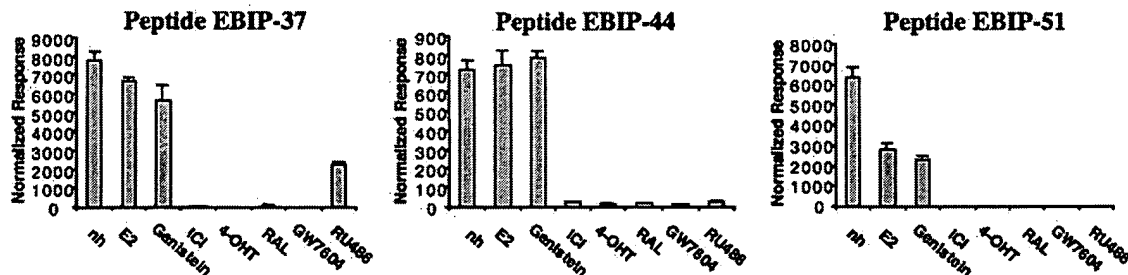
SRC-1 and GRIP1 interacted with ER β in the absence of hormone (data not shown).

When the ER β -selective peptides were analyzed in this assay, we were able to divide them into two classes: those that interacted with the receptor in the absence of hormone (Fig. 2B) and those whose interaction was strictly agonist dependent (Fig. 2C). The profiles of three representative peptides from each group are shown. Several of the peptide fusions (ER β -interacting peptides EBIP-37 and EBIP-44) interacted equally well with ER β in the absence of hormone and

A. Controls



B. Hormone-Independent



C. Hormone-Dependent

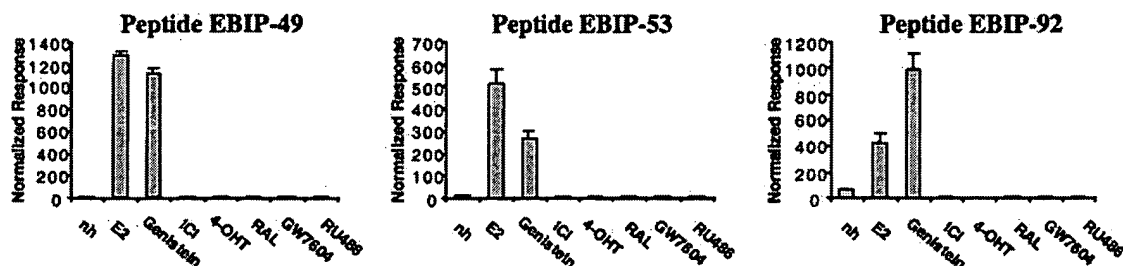


Fig. 2. ER β -Selective LXXLL Peptides Display Hormone Specificity

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ER β in the presence of different ER ligands. HepG2 cells were transiently transfected with the pVP16-ER β expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV- β -gal control plasmid. After transfection, cells were treated with vehicle (nh) or either 10^{-7} M 17 β -estradiol (E₂), ICI182,780 (ICI), 4-hydroxytamoxifen (4OH-T), raloxifene (RAL), GW7604, RU486, or 10^{-6} M genistein. After 24 h cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV- β -gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%. A, The SRC-1 NR-box and GRIP1 NR-box were used as controls. Based upon the pattern of interactions, the peptides were divided into two classes: B, hormone-independent (six peptides) and C, hormone-dependent (nine peptides). The activities of three representative peptides are shown in both parts B and C, and these illustrate the three different patterns of interactions observed in each case.

in the presence of agonist (Fig. 2B). However, these interactions were antagonized by antiestrogen administration. Interestingly, peptide EBIP-51 interacted most efficiently with apo-ER β , suggesting the existence of cofactors that prefer to associate with ER β in a constitutive manner (see *Discussion*). The binding of LXXLL motifs to ER β in the absence of hormone was puzzling, in view of the fact that it was previously observed that the human ER β does not demonstrate ligand-independent transcriptional activity (11). This result suggests that the aporeceptor may recruit cofactors in some contexts, but that this interaction is not transcriptionally productive. In these studies, we also observed that the antiprogesterin RU486 was able to decrease interactions between the peptides and receptor in these assays, a result that agrees with recent reports that RU486 can function as an ER β antagonist (23).

Nine of the peptide fusions studied were found to interact only with agonist-activated ER β . Interestingly, within this class, three distinct binding patterns were observed (Fig. 2C). Specifically, peptide EBIP-92 appeared to interact more efficiently with genistein-activated ER β than that activated by 17 β -estradiol, whereas the estradiol-activated receptor interacted equally as well with EBIP-49, and more efficiently with EBIP-53. These data, indicating that genistein and estradiol do not function in the same manner when assayed on ER β , were interesting in light of the unique functional properties that have recently been ascribed to genistein (8, 24). Similar differences in efficacy were noted when the experiments were repeated over a full range of ligand concentrations (data not shown). These results suggest that estradiol and genistein induce unique conformational changes within ER β . This hypothesis is supported by recent crystallography studies that showed that ER β helix 12 (AF-2) assumes

a distinct position when occupied by genistein compared with the distinctive agonist position observed for helix 12 of the estradiol-bound ER α (16, 25). If the estradiol-ER α crystallographic data are extrapolated to ER β , it is possible that genistein-liganded ER β interacts with cofactors in a different manner than the estradiol-activated receptor or that the former complex recruits a unique coactivator in some settings.

Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ER β

One of the most interesting classes of peptides identified in this study were those that interacted with ER β in a constitutive manner but that were unable to interact with the receptor when occupied by antagonists. A trivial explanation for these observed binding characteristics was that these peptides had a higher affinity for ER β than the peptides that required ligand. Alternatively, these peptides may interact in a unique manner with the coactivator binding pocket within ER β . These different possibilities were tested using a quantitative phage ELISA. Specifically, the phage stocks of each peptide-expressing clone were titered, and then the binding of different concentrations of phage expressing peptides to ER β was tested in the presence and absence of estradiol. The results of this analysis, shown in Fig. 3, indicated that even at the lowest input phage concentrations, the hormone-independent peptides maintained their ligand-independent interactions with ER β ; two representative peptides are shown. This was in contrast to the hormone-dependent peptides, which maintained their estradiol-dependent interaction with ER β throughout the range of phage concentrations (representative example shown in Fig. 3C). Neither the absolute numbers of phage binding

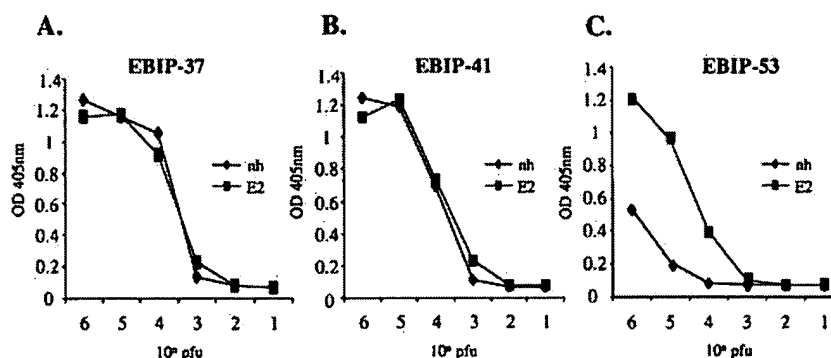


Fig. 3. Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ER β

The binding of each titered phage clone to ER β was measured by ELISA. Purified ER β protein was added to 96-well plates, and 50 μ l of each phage stock or serial dilutions were added to individual wells and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-6} M 17 β -estradiol. The wells were washed with PBST to remove nonbinding phage and incubated with a horseradish peroxidase-conjugated anti-M13 antibody. Immunocomplexes were detected with ABTS (2', 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H₂O₂. The colorimetric change was quantitated by measuring the absorbance at 405 nm. The binding patterns of two representative peptides from the hormone-independent class (A and B), and the binding of one representative peptide from the hormone-dependent class (C) are shown.

to each target nor the apparent binding affinity were significantly different. These results indicate that receptor-binding affinity is not what distinguishes peptides that interact with ER β in a ligand-dependent manner from those that bind constitutively, but rather these classes of LXXLL-containing peptides interact in different ways with the ER β -coactivator binding pocket.

Disruption of ER β Transcriptional Activity by LXXLL-Containing Peptides

The ability of the ER β -interacting peptides to function as ER subtype-selective antagonists in target cells was next assessed. This was accomplished using transcriptional interference assays in mammalian cells transfected with either ER α or ER β , together with an empty Gal4DBD vector (pM) or Gal4DBD fusions of the peptides. The EBIP-37, EBIP-41, and EBIP-45 peptides were used in these initial experiments, because they appeared to interact most efficiently with ER β in the mammalian two-hybrid system (Fig. 1A). Under the conditions of this assay, we noted that the transcriptional activity of ER α was unaffected by overexpression of the ER β -selective peptides (Fig. 4A). Coexpression of the GRIP1 NR-box sequence (three LXXLL motifs), however, inhibited ER α transcriptional activity by 77%, thus validating the use of LXXLL peptides as antagonists of ER transcriptional responses. When the effect of our peptides on ER β activity was assessed, we observed that all of the LXXLL sequences functioned as effective antagonists (Fig. 4B). Significantly, coexpression of peptide EBIP-37 with ER β resulted in 100% inhibition of the transcriptional response, whereas the EBIP-41, EBIP-45, and GRIP-1 (NR-box) peptide fusions produced 85, 70, and 90% inhibition, respectively. Western immunoblotting revealed that

expression of the peptides did not alter the cellular levels of ER β (data not shown). These results indicate that it is possible to target ER β -coactivator interactions in a selective manner and validate this general approach to develop antagonists of additional members of the nuclear receptor superfamily.

ER β -Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

One of the major objectives of this study is to develop highly specific inhibitors that can be used to evaluate the relative contributions of the two ER subtypes in estrogen signaling. Consequently, we next examined the ability of the 15 ER β -selective peptides to interact with other members of the nuclear receptor superfamily. This was accomplished using the mammalian two-hybrid assay to examine interactions of each peptide with ER α , ER β , and 11 different nuclear receptors. Table 1 summarizes the interactions observed between each of the peptides and receptors in the presence of their cognate ligands. No clear pattern emerged from these studies, as each receptor appeared to exhibit distinct peptide binding preferences. Based on these results, it is possible that most of the receptors will eventually be found to display different cofactor preferences. The observation that the androgen receptor (AR) did not interact with any of the peptides tested and that ROR α interacted with only two of the 15 peptides suggest that these receptors might have very specific coactivator requirements.

Interestingly, the peptides identified in our screen using unliganded ER β as bait (EBIP-37, EBIP-41, EBIP-44, EBIP-45), and which interacted with the receptor in an agonist-independent manner in mammalian cells (Fig. 3), were found to interact with most of

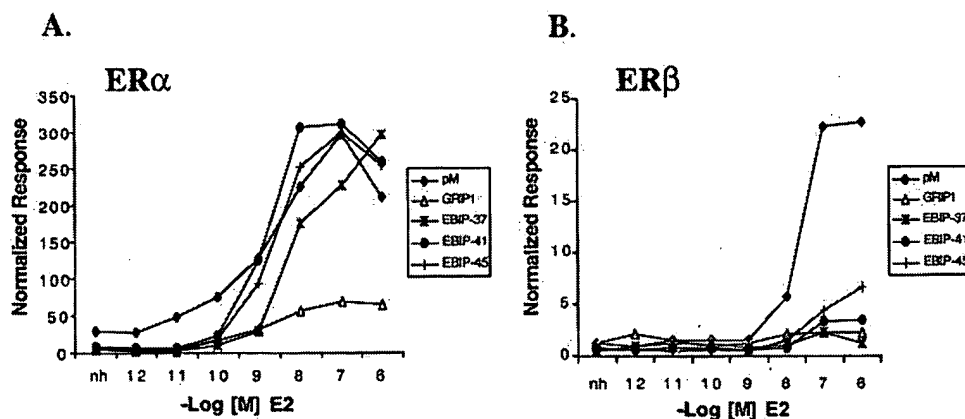


Fig. 4. Disruption of ER β Transcriptional Activity by LXXLL-Containing Peptides

The effects of three LXXLL-containing peptides (EBIP-37, EBIP-41, and EBIP-45) on ER α and ER β transcriptional activity were examined. A peptide containing the LXXLL motifs (NR box) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with either the ER α (A) or ER β (B) expression vector, together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc reporter and pCMV- β -gal control plasmid. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17 β -estradiol (E $_2$) for 24 h, and luciferase assays were performed. Each value was normalized to the β -galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

Table 1. ER β -Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

	ER α	ER β	PR-A	PR-B	GR	AR	RAR α	RXR α	TR β	VDR	ROR α	LXR	FXR
SRC-1	+	+	+	+	+	—	+	+	+	+	—	+	+
GRIP1	+	+	+	+	+	—	+	+	+	+	—	+	+
EBIP-37	—	+	+	+	+	—	+	+	+	+	+	+	+
EBIP-41	—	+	—	—	+	—	+	+	+	—	—	+	—
EBIP-44	—	+	+	+	+	—	+	+	+	—	—	+	+
EBIP-45	—	+	+	+	—	—	—	+	+	—	—	+	—
EBIP-49	—	+	—	—	+	—	—	—	—	—	—	—	—
EBIP-51	—	+	—	—	—	—	—	+	+	+	—	+	—
EBIP-53	—	+	+	+	+	—	—	+	+	—	—	+	—
EBIP-56	—	+	—	—	—	—	—	—	—	—	—	—	—
EBIP-60	—	+	+	+	+	—	—	+	—	—	—	+	—
EBIP-66	—	+	+	+	+	—	—	+	—	—	—	—	—
EBIP-70	—	+	—	—	—	—	+	+	+	+	—	+	—
EBIP-76	—	+	—	—	—	—	—	+	+	—	—	+	—
EBIP-87	—	+	+	+	+	—	+	+	+	—	—	+	—
EBIP-92	—	+	—	—	—	—	—	—	—	—	—	—	—
EBIP-96	—	+	+	+	+	—	—	—	+	+	+	+	—

The ability of the ER β -selective LXXLL motifs to interact with several nuclear receptors was tested in the mammalian-two-hybrid assay (+ denotes interaction; — denotes lack of interaction). ER β -specific peptides are indicated in *bold*. Shown here are the interactions that form between the peptides and receptors in the presence of the receptor-specific agonists, with the exception of ROR α , which is constitutively active. An interaction (+) was defined by the observation of a statistically significant increase in activity when both the receptor and peptide were coexpressed compared to that present when either was cotransfected with the parent vector of the other. HepG2 cells were transiently transfected with each pVP16-receptor fusion expression vector in combination with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV- β -gal control plasmid. Each receptor expression construct includes the VP16 activation domain sequence fused 5' to the entire coding sequence for the human form of the respective receptor, with the exception of pVP16-FXR, which contains the rat FXR homolog. Following transfection, cells were treated with vehicle or the following hormones: 10^{-7} M 17β -estradiol for ER α and ER β , 10^{-7} M progesterone for PR-A and PR-B, 10^{-7} M dexamethasone for GR, 10^{-6} M 5α -dihydrotestosterone for AR, 10^{-7} M 9-*cis*-retinoic acid for RAR α and RXR α , 10^{-7} M T $_3$ for TR β , 10^{-7} M 1,25-dihydroxyvitamin D $_3$ for VDR, 10 μ M 22R-hydroxycholesterol for LXR, and 50 μ M chenodeoxycholic acid for FXR. After 24 h luciferase assays were performed, and each value was normalized to the β -galactosidase activity; — indicates that no significant increase in activity was observed when the receptor and peptide were coexpressed.

the nuclear receptors tested. The most important result, however, was that EBIP-56 and EBIP-92 interacted exclusively with ER β and did not interact with any other receptor under the conditions tested. Other peptides, such as EBIP-87 and EBIP-96, were found to interact with multiple receptors. Cumulatively, the results of these studies indicate that it is possible to identify LXXLL-containing peptides that interact in a highly specific manner with ER β .

Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ER β

The antagonist efficacy of the ER β -specific peptides, EBIP-56 and EBIP-92, was next evaluated. The nuclear receptor interaction regions of most of the well validated coactivators have been shown to contain multiple LXXLL domains, which facilitate the interaction of these proteins with the AF-2 coactivator binding pocket of their targeted receptor (18). Reflecting this observation, we created two-copy Gal4DBD fusions of our peptides. The two LXXLL motifs were separated by sequences corresponding to the linker region between NR-box 2 and NR-box 3 of GRIP1.

When expressed in mammalian cells, we observed that while the GRIP1 NR-box sequences inhibited the activity of ER α by 60%, the peptides 2xEBIP-56 and 2xEBIP-92 had no effect on transcriptional response (Fig. 5A). However, when tested on ER β , it was found that the 2xEBIP-56 and 2xEBIP-92 peptides suppressed estrogen-stimulated transcriptional activity by 82% and 97%, respectively (Fig. 5B). Similar results were obtained using 1xEBIP-56 and 1xEBIP-92, although higher levels of expression of these peptides were required to attain the same degree of antagonism as their dimeric counterparts. Western immunoblotting was used to demonstrate that expression of these peptides did not alter cellular levels of ER β (data not shown). Thus, ER β -specific LXXLL-containing peptides can function as potent inhibitors of ER β transcriptional activity.

ER β -Specific LXXLL-Containing Peptides Disrupt the Transcriptional Activity of ER α /ER β Heterodimers

We and others have demonstrated that the transcriptional activity of agonist-activated ER α is significantly greater than ER β in most cell and promoter contexts

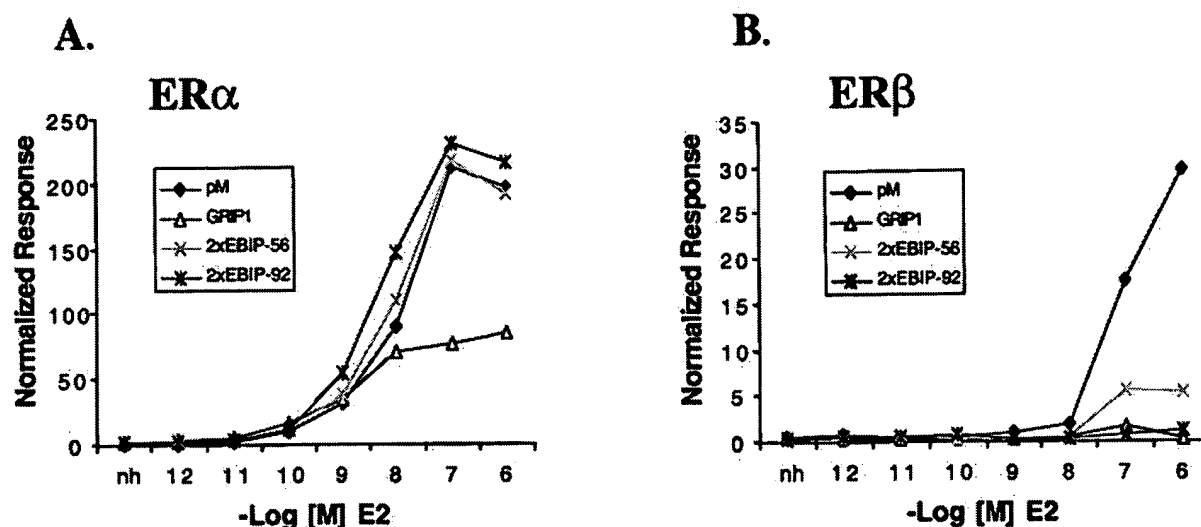


Fig. 5. Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ER β

The ability of the ER β -specific LXXLL peptides EBIP-56 and EBIP-92 to disrupt the transcriptional activities of ER α and ER β was examined. For these experiments, constructs containing two copies of the peptides (2x-EBIP-56 and 2x-EBIP-92) were used. A peptide containing the three LXXLL motifs (NR-boxes) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with the ER α (A) or ER β (B) expression vector together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc and pCMV- β -gal plasmids. After transfection, cells were treated with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17 β -estradiol (E $_2$) for 24 h, and luciferase assays were performed. Each value was normalized to the β -galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

(7, 9–11). Not surprisingly, therefore, in cells engineered to produce both ER subtypes, the overall response to estradiol is reduced. This suggests that one of the functions of ER β is to modulate ER α transcriptional activity in target cells. At subsaturating concentrations of agonist, ER β completely suppresses ER α transcriptional activity, whereas no inhibition is observed when the assay is performed in the presence of saturating concentrations of 17 β -estradiol (11). These findings suggest that the role of ER β in estrogen signaling is complex. Previously, we have shown that ER β is bound constitutively to DNA in the absence of hormone (11). Consequently, at subsaturating concentrations of hormone, the receptor is capable of competitively inhibiting the activity of ER α homodimers and ER α /ER β heterodimers by blocking their ability to interact with target gene promoters. Under saturating concentrations of hormone, we have proposed that ER α /ER β heterodimers form, and that the functional properties of this complex are similar to that of an ER α homodimer. However, without a specific ER β antagonist, it has not been possible to prove that the heterodimeric ER α /ER β complex was functionally active in cells.

The identification of specific peptide antagonists of ER β has enabled us to evaluate the functional significance of ER α /ER β heterodimers. This was accomplished by determining the impact of expressing the 2xEBIP-92 antagonist in cells and examining its impact on ER α and ER β -mediated transcriptional activity. The results of this analysis are shown in Fig. 6. As

observed previously, expression of 2x-EBIP-92 in cells had no effect on ER α transcriptional activity, whereas it completely suppressed ER β activity. Importantly, however, in cells expressing both receptor subtypes it was demonstrated that the ER β -specific antagonist, 2xEBIP-92, was capable of significantly reducing the transcriptional activity of the ER α /ER β heterodimer. To rule out the possibility that, in the context of the heterodimer, the ER β -specific peptides may interact directly with ER α we used mammalian two-hybrid assays to assess the interaction of the heterodimeric complexes with a subset of the EBIPs. For this purpose, we created an ER β mutant that disrupts AF-2 but that has no effect on the receptor's ligand binding characteristics. As shown in Fig. 7A, an ER β -VP16, but not an ER β -3x-VP16, chimera was able to interact with the LXXLL peptides when tested in the two-hybrid assay. Since ER β -3x heterodimerizes with ER α in a manner that was indistinguishable from ER β it was possible to use this mutant to test the interaction of the ER β -interacting peptides with the ER α / β heterodimer (11). First, we demonstrated that expression of ER α in target cells had no effect on the ability of ER β to interact with EBIPs 37, 41, 56, or 92 (Fig. 7B). Although it would be difficult to quantitate the amount of heterodimer formed under the conditions of this assay, the results suggest that ER β specificity is maintained in this context. The most important result, however, was that in cells expressing ER α , the ER β -3x-VP16 chimera was inactive in the two-hybrid assay (Fig. 7B). Given the characteristics of the ER β -3x chimera, a

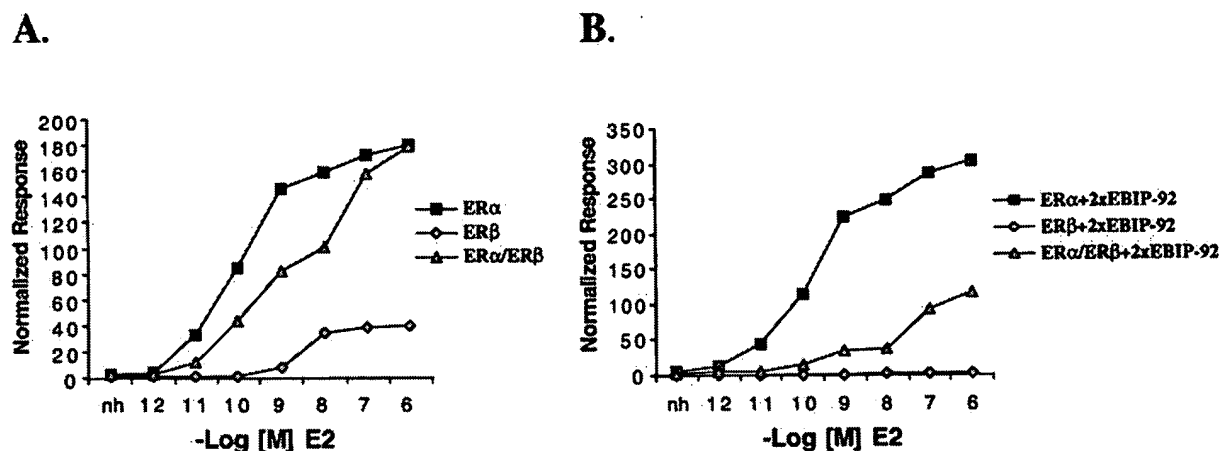


Fig. 6. ER β -Specific LXXLL Containing Peptides Disrupt the Transcriptional Activity of ER α /ER β Heterodimers

HepG2 cells were transiently transfected with either the ER α or ER β expression vectors, or equal quantities of both vectors together with the pM (Gal4DBD) empty vector (A) or the 2xEBIP-92 peptide-Gal4DBD fusion construct (B) and the 3x-ERE-TATA-Luc and pCMV- β -gal plasmids. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17 β -estradiol (E₂) for 24 h, and luciferase assays were performed. Each value was normalized to the β -galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

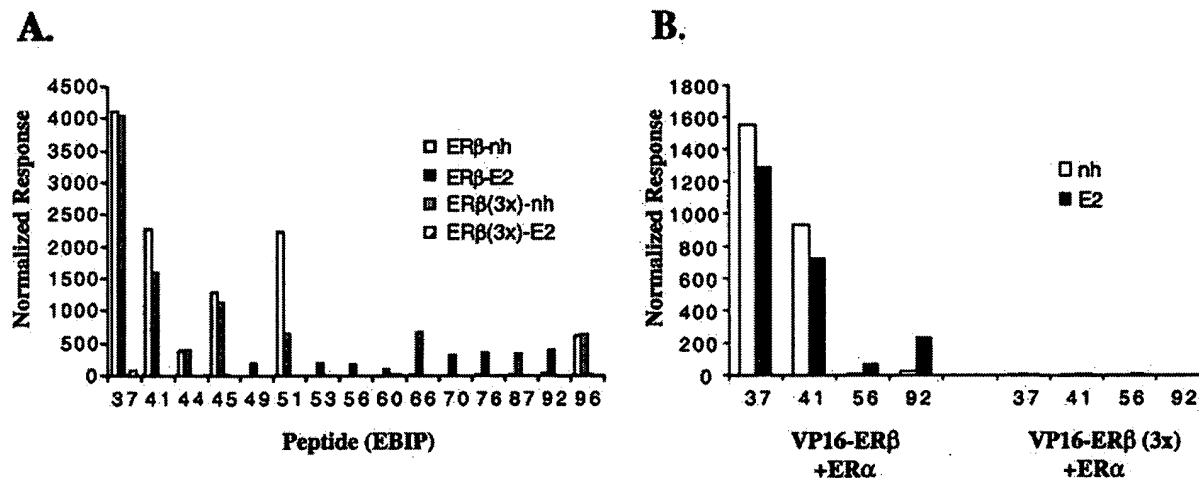


Fig. 7. The Specificity of the ER β -Interacting Peptides Is Maintained within the Context of an ER α /ER β Heterodimer

A, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ER β or ER β (3x). ER β (3x) contains three point mutations introduced into AF-2, which nullify the AF-2 activity of the receptor. HepG2 cells were transiently transfected with the pVP16-ER β or pVP16-ER β (3x) expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV- β -gal control plasmid. B, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ER α /ER β heterodimers or ER α /ER β (3x) heterodimers. The interaction of four representative peptides are shown. HepG2 cells were transiently transfected with the pVP16-ER β or pVP16-ER β (3x) expression vector and an ER α expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV- β -gal control plasmid. After transfection, cells were treated with vehicle (nh) or 10^{-7} M 17 β -estradiol (E₂). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV- β -gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

positive interaction in the two-hybrid assay would only have been possible if the peptides were able to interact with the coactivator binding pocket of ER α . We conclude from this experiment that even within the context of an ER α /ER β heterodimer that the ER-interacting characteristics of the EBIPs are main-

tained. Therefore, the inhibitory effects of 2xEBIP-92 on the ER α /ER β heterodimer is mediated by blocking ER β function. Thus, although it has been shown previously that ER α and ER β preferentially form heterodimers when coexpressed (26, 27), we now demonstrate ER β contributes in a positive

manner to the overall activity of the complex under agonist saturating conditions.

Comparison of the Amino Acid Sequences of the ER β -Interacting Peptides

Alignment of the three classes of ER β -interacting peptides identified (hormone-independent, hormone-dependent, and ER β -specific) was next performed (Table 2). Surprisingly, minimal homology was observed outside of the conserved LXXLL motif between members within the same class. However, we observed that the two ER β specific peptides, EBIP-56 and EBIP-92, contain a tryptophan residue at position -5 that was not found in any of the other 68 ER β -interacting peptides that were isolated in the primary screen. Therefore, we postulated that the tryptophan influenced the specificity of the peptide-ER β interactions. To test this hypothesis, the tryptophan residue in the EBIP-92 peptide was converted to glutamine (an amino acid found at this position in many of the peptides identified). Analysis using a mammalian two-hybrid revealed that while the wild-type EBIP-92 interacted with ER β in a hormone-dependent manner, a variant peptide in which the tryptophan residue at position -5 was mutated was unable to interact with ER β (data not shown). These studies demonstrate the importance of the sequences surrounding LXXLL motifs in determining receptor selectivity and suggest that it may be possible to use site-directed mutagen-

esis to optimize the interactions of the peptides identified with their protein targets.

DISCUSSION

Development of ER Peptide Antagonists

The most important outcome of this series of studies was the identification of highly potent, specific ER β antagonists. Using these peptides, it is possible to efficiently inhibit ER β transcriptional activity by disrupting interactions between the receptor and cellular coactivators. These reagents are important tools that will facilitate an evaluation of the role of this ER subtype in estrogen signaling. For instance, we have used these peptides to demonstrate that ER α /ER β heterodimeric complexes can form within cells, and that ER β contributes in a positive manner to the overall activity of the estrogen-activated complex.

Recently, it has been suggested that the two ER subtypes may oppose the actions of each other in target organs. Although controversial, this hypothesis is supported by the observation that the β ERKO mouse displays epithelial hyperplasia in the prostate and bladder (14), an increase in bone mineral content (28), and an increased responsiveness to estrogen in the uterus (29), reflecting possibly an enhancement of ER α -mediated transcriptional activity. Using an appropriate delivery system, it may be possible to antagonize ER β action using the receptor-specific peptides and test directly the hypothesis that ER β functions as an ER α modulator in some tissues.

It has recently been shown that both ER subtypes are expressed in breast tumors (30–32) and that ER β expression is up-regulated in tumors that have developed tamoxifen resistance (32). Thus, there is an unmet medical need to develop novel ER antagonists as 1) potential breast cancer therapeutics and 2) tools to specifically define the role of ER β in breast cancer cell biology. The finding that none of the LXXLL-containing sequences in this study interact with antiestrogen-liganded receptor suggests that suitably formulated ER peptide antagonists could be coadministered with tamoxifen to completely block estrogen-stimulated proliferative pathways in the breast, using two mechanistically distinct modes of antagonism. Recent studies provide evidence that tamoxifen resistance in breast tumors may arise from the up-regulation of coactivator proteins, which may permit cells to recognize tamoxifen as an agonist and growth stimulant (22). The identification of peptides that disrupt receptor-coactivator interactions provides a novel mechanism by which the mitogenic actions of activated ER can be blocked in both antiestrogen-responsive and -resistant breast cancer cells. Theoretically, the peptide antagonists that we have identified could be developed as second line pharmaceutical treatments for ER-positive, tamoxifen-refractory tumors.

Table 2. Comparison of the Amino Acid Sequences of the ER β -Interacting Peptides

	-3-2-1	LXXLL	+1+2+3
ER β -selective peptides			
Hormone-independent			
Peptide EBIP-37	TGGGVSL	LLHLL	NTEQGES
Peptide EBIP-41	RRDDFPL	LISLL	KDGALSQ
Peptide EBIP-44	YGLKMSL	LESLL	REDISTV
Peptide EBIP-45	MSYDMLS	LYPLL	TNSLLEV
Peptide EBIP-51	FPAEFPL	LTYLL	ERQGMDE
Peptide EBIP-96	VESEFPY	LLSLL	GEVSPQP
Hormone-dependent			
Peptide EBIP-49	VSSEGRL	LIDLL	VDGQQSE
Peptide EBIP-53	DTPQSPL	LWGLL	SSDRVEG
Peptide EBIP-60	GGTQDGY	LWSLL	TGMPEVS
Peptide EBIP-66	SLPEEGF	LMKLL	TLEGDAE
Peptide EBIP-70	VMGNNPI	LVSLL	EEPSEEP
Peptide EBIP-76	VLVEHPI	LGGLL	STRVDSS
Peptide EBIP-87	QTPL	LEQLL	TEHIQQG
ER β -specific peptides			
Peptide EBIP-56	GSWQDSL	LLQLL	NRTELMA
Peptide EBIP-92	SVWPGPE	LLKLL	SGTSVAE

The ER β -selective peptides were divided into two classes: hormone-independent and hormone-dependent. Also shown are the sequences of the ER β -specific peptides EBIP-56 and EBIP-92, which constitute a third class of ER β -interacting peptides. The conserved tryptophan at position -5 relative to the LXXLL motif in these two sequences is shown in *bold*.

Previous studies in our laboratory (20) reported the identification of the ER β selective peptide 293. However, while the peptide displays selectivity for ER β over ER α , 293 was found to interact with many of the other nuclear receptors. In this study our goal was to develop peptides that interacted in a completely specific manner with ER β , which was accomplished in the discovery of peptides EBIP-56 and EBIP-92. A similar study was recently reported (33) in which a panel of LXXLL-containing peptides were identified that demonstrated selectivity for ER β over thyroid hormone receptor (TR). However, the authors of that study indicated that most of their ER β -interacting peptides cross-react with ER α . Thus, EBIP-56 and EBIP-92 represent the only reagents available that can be used to specifically inhibit ER β transcriptional activity.

Ligand-Independent Recruitment of LXXLL Motifs

One of the most important findings of this study was that the unliganded ER β is capable of recruiting many of the LXXLL peptides. Interestingly, studies with ER α showed that LXXLL-containing sequences were capable of a low but significant basal level of interaction in the absence of hormone (20). These results suggested that a fraction of the ER α molecules in a cell might reside in an active conformation, thus permitting recruitment of LXXLL motifs in the absence of receptor agonists. This may explain why ER α can activate transcription in some contexts in the absence of hormone. Surprisingly, although apo-ER β is capable of binding several different LXXLL-containing peptides, this form of the receptor does not activate transcription in the absence of agonist (11). Consistent with this observation, we have shown using *in vitro* protein-protein interaction studies that ER β , but not ER α , can bind to GRIP1 in the absence of ligand (our unpublished results). One possibility is that the ER β aporeceptor is present in an inhibitory complex containing both coactivators and corepressors, and that the binding of hormone enhances the functionality of associated activators and promotes the dissociation of repressor proteins. Alternatively, unliganded ER β may bind to some cofactors in a manner that is not transcriptionally productive. An activity of this nature has not yet been demonstrated for ER β ; however, it has been shown that unliganded peroxisome proliferator activated receptor- γ (PPAR γ) interacts with the coactivator PGC-1 (PPAR γ coactivator 1, a protein that has no apparent coactivator activity), and this protein is responsible for recruiting SRC-1 when agonist is added (34).

The ability of nuclear receptors to interact with LXXLL motifs in their apo- state raises the possibility that ligand regulation of coactivator recruitment may have evolved to enable receptor activity to respond to changes in cellular homeostasis. Consistent with this hypothesis is the observation that several orphan receptors [estrogen-receptor-related proteins

(ERR1, ERR2, and ERR3)] that have not yet been shown to require ligands bind SRC-1, GRIP1, or activator of thyroid receptor (ACTR) in a ligand-independent manner (35, 36). Similarly, the orphan receptor 1/ retinoid X receptor (OR1/RXR) heterodimer is capable of ligand-independent cofactor recruitment (37). Recent studies have also illustrated that ligand-independent signaling pathways can result in activation of ER β by promoting agonist-independent coactivator binding (38). These observations suggest that the general mechanisms of hormone-dependent and independent transcriptional activation by nuclear receptors may be similar, and that in some cases the role of ligand may be as a catalyst, but not as a required part of receptor activation. Thus, ER β may be a receptor whose state of evolution is intermediate between the orphan receptors and the more classical steroid receptors.

Nuclear Hormone Receptors Have Distinct Preferences for LXXLLs

A recurring theme in these studies is that nuclear receptors have distinct preferences for LXXLL motifs. Previous work in our laboratory using peptide display has demonstrated that the sequences flanking the core LXXLL domain are important determinants of receptor selectivity (20). Mutagenesis studies have also been used to identify residues important for both receptor binding affinity and specificity (18, 39). McInerney *et al.* demonstrated that of the three helical LXXLL-containing regions of SRC-1, a single helical domain was sufficient for ER activation, whereas a combination of two distinct helical regions were required for PR, TR, retinoic acid receptor (RAR), and PPAR γ actions (40). These studies indicate that different receptors can interact with the same cofactor in different ways.

To complement these previous studies, we observed that peptide EBIP-37 (identical to an LXXLL motif in RIP140) interacted selectively with ER β , but not ER α . Therefore, since RIP140 can bind to and repress the transcriptional activities of both ER α and ER β (Ref. 41 and our unpublished data), it is likely that each receptor subtype utilizes distinct LXXLL motifs within this factor, enabling them to bind. The observation that each of the receptors examined in our study displayed a unique pattern of interaction with LXXLL peptides also provides evidence that the receptors may bind different coactivators, or alternatively, recruit the same factors by utilizing distinct binding regions. It is likely therefore, that it will be possible to develop LXXLL-containing antagonists for many of the nuclear receptors. It was surprising, given the structural conservation among the nuclear receptors and associated cofactors, that peptides could be identified which block these interactions in a highly specific manner. However, given that it has been possible to develop specific ER β antagonists

using this approach, we believe that it will be feasible to identify inhibitors of a wide variety of transcription factors by interfering with specific protein-protein interactions.

MATERIALS AND METHODS

Biochemicals

DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin Elmer Corp. (Norwalk, CT) or Promega Corp. 17 β -Estradiol, genistein, 4-hydroxytamoxifen, 9-*cis*-retinoic acid, dexamethasone, 5 α -dihydrotestosterone, T₃, progesterone, 22R-hydroxycholesterol, and chenodeoxycholic acid were purchased from Sigma (St. Louis, MO). RU486 was a gift from Ligand Pharmaceuticals, Inc. (San Diego, CA). The estrogen receptor antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. Eric Larsen (Pfizer, Inc., Groton, CT). GW7604 was a gift from Dr. Tim Willson (Glaxo-Wellcome, Research Triangle Park, NC); 1,25-dihydroxyvitamin D₃ was purchased from Duphar Pharmaceuticals (Dawesep, The Netherlands). The mouse monoclonal anti-Gal4DBD antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal ER β antibody was a gift from Dr. Geoffrey Greene (University of Chicago, Chicago, IL). Secondary antibodies, Hybond-C extra transfer membranes, and ECL reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Affinity Selection of ER β -Binding Peptides

Baculovirus-expressed human ER β (amino acids 1–477) was purchased from PanVera Corp. (Madison, WI). ER β (4 pmol) was added to 100 μ l of NaHCO₃, pH 8.5, in single wells of a 96-well Immulon 4 plate (Dynex Technologies, Inc.). The protein was then incubated in the absence or presence of 10^{−6} M 17 β -estradiol overnight at 4 °C. A duplicate well containing BSA alone was used as a control. The wells were blocked with 150 μ l of 0.1% BSA in NaHCO₃ for 1 h at room temperature and then washed five times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3, 0.1% Tween 20). Twenty five microliters of the phage library (>10¹⁰ phage) were preincubated on ice for 1 h in 125 μ l PBST, 0.1% BSA, and 10^{−6} M 17 β -estradiol or vehicle. The phage library was added to the wells, and the plate was sealed and incubated at room temperature for 8 h with gentle agitation. The wells were washed five times with PBST to remove nonbinding phage. The binding phage were eluted with 100 μ l of 50 mM glycine-HCl, pH 2.0 (prewarmed to 50 °C), and subsequently eluted with 100 μ l of 100 mM ethanolamine, pH 11.0. The first eluant was neutralized with 200 μ l of 200 mM Na₂HPO₄, pH 8.5, before being combined with the second eluant. The bound phage were amplified in DH5 α F⁺ cells for 6 h and recovered by centrifugation. The amplified phage were used for subsequent rounds of panning. Three rounds of panning were performed. The enrichment of ER β binding phage in each round of panning was confirmed by ELISA. Individual phage clones were purified after the third round of panning. The single-stranded phage DNA was isolated from each clone, and the peptide sequences were determined by DNA sequencing.

ELISA

Purified ER β protein (0.4 pmol) was added to 96-well Immulon 4 plates as detailed above. Fifty microliters of each purified phage were added to an individual well and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{−6} M of various ER ligands. The wells were washed five times with PBST to remove nonbinding phage. The binding of each peptide to full-length ER α (provided by Panvera Corp.) in the presence of various ER ligands was also tested in this assay. A horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted 1:5000 in PBST, 100 μ l of the mixture was added to each well, and the solutions were incubated for 1 h at room temperature. The wells were washed five times with PBST, and immunocomplexes were detected with ABTS (2',2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H₂O₂. The colorimetric change was quantitated by measuring the absorbance at 405 nm on a plate reader (Multiskan MS; Labsystems, Marlboro, MA).

Plasmids

The Gal4DBD-peptide fusions were constructed as follows. The peptides were excised from the mBAX phage vectors with *Xba*I and *Xho*I. The parent pMx vector (20) (containing the Gal4DBD) was digested with *Sal*I and *Xba*I. The peptides were then ligated in frame to the pMx vector, creating Gal4DBD-peptide fusion constructs. The constructs containing two copies of the LXXLL-containing peptides (2x-EBIP-56 and 2x-EBIP-92) were created as follows: pM-EBIP-56 and pM-EBIP-92 were digested with *Xba*I. The linker region between the second and third LXXLL motifs within the GRIP1 cDNA was amplified by PCR, digested with *Nhe*I and *Xba*I, and ligated into pM-EBIP-56 and pM-EBIP-92, at the 3' of the peptide coding region. These vectors were then digested with *Sal*I and *Xba*I and an oligonucleotide encoding a second copy of the peptide was inserted into these sites 3' to the GRIP1 linker. The construction of pM-SRC-1 (NR-box) and pM-GRIP1 (NR-box) has been described previously (20).

The mammalian expression plasmid for the peptide EBIP-92 mutant was constructed by site-directed mutagenesis as follows. The pM-EBIP-92 vector was used as the template, and a point mutation in the conserved tryptophan residue was created using PCR-based oligonucleotide-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA). The sequences of the oligonucleotides used for PCR were 5'-CTCGAGAAGTGT-TGAGCCGGGTCCGGAGCTGCTTAAGCTGCTGTCGGGGA-CGAGTGTGGCGGAG (forward) and 3'-CTCCGCCACAC-TCGTCCCCGACAGCAGCTTAAGCAGCTCCGGACCCGGC-TCAACACTTCTCGAG (reverse).

pVP16ER α , pVP16ER β , pVP16RAR α , and pVP16RXR α have been described previously (20). VP16GR, VP16PR-A, VP16PR-B, and VP16AR expression plasmids were gifts from J. Miner (VP16GR), D. X. Wen (VP16PR-A and VP16PR-B), and K. Marschke (VP16AR) (Ligand Pharmaceuticals, Inc., San Diego, CA). VP16VDR was a gift of J. W. Pike (University of Cincinnati, Cincinnati, OH), and the VP16TR β expression plasmid (pCMX-VP-F-hTR β) was provided by D. D. Moore (Baylor College of Medicine, Houston, TX). pVP16ROR α -LBD was a gift from A. R. Means (Duke University Medical Center, Durham, NC). The cDNAs for the human liver X receptor (LXR) and rat farnesoid X receptor (FXR) were provided by D. J. Mangelsdorf (University of Texas, Dallas, TX). pVP16LXR, and pVP16FXR were created as described previously for the other nuclear receptor VP16 fusions (20).

The mammalian expression plasmids for ER α (pRST7ER) and ER β (pRST7ER β) have been described previously (11, 42). The reporter 5x-GAL4-TATA-Luc (a gift from Dr. Xiao-Fan Wang, Duke University Medical Center) contains five palin-

dromic copies of the GAL4 transcription factor response element cloned into pGL2-TATA-Inr (Stratagene). The 3x-ERE-TATA-Luc reporter contains three copies of the vitellogenin ERE (43).

All of the PCR-based constructs were sequenced to verify the accuracy of the amplified sequences.

Cell Culture and Transient Transfection Assays

HepG2 cells were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS (Life Technologies, Inc.), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h before transfection. DNA was introduced into the cells using lipofectin (Life Technologies, Inc.). Triplicate transfections were performed using 3 μ g of total DNA. In standard mammalian two-hybrid assays, 1,500 ng of reporter (5x-GAL4-TATA-Luc), 500 ng of receptor-VP16 fusion, 500 ng of pM (Gal4DBD)-peptide fusion constructs, 100 ng of the pCMV- β gal normalization vector (44), and 400 ng of the control vector pBSII-KS (Stratagene) were used. For receptor disruption studies, 1,500 ng of reporter (3x-ERE-TATA-Luc), 250 ng of receptor (either pRST7ER α or pRST7ER β), 1000 ng of pM-peptide fusion constructs or the parent pM vector, 100 ng of pCMV- β gal, and 150 ng of pBSII-KS were used. Cells were incubated with the DNA/lipofectin mix for 3 to 6 h and then washed with PBS and the transfection mix was replaced with phenol red-free MEM containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT). The receptor ligands were added to the cells 20–24 h before the assays. Luciferase and β -galactosidase assays were performed as described previously (45). All experiments were repeated a minimum of three times.

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